APPENDIX A

TO THE

RESPONSE TO OFFICE ACTION

FILED NOVEMBER 11, 2011

IN THE MATTER OF EUROPEAN PATENT APPLICATION 04 709 824.9, IN THE NAME OF SHIRE HUMAN GENETIC THERAPIES, INC.

DECLARATION

1. I am Prof. Dr. Thomas Dierks, currently of Universität Bielefeld, Fakultät für Chemie, Universitätstr. 25, 33615 Bielefeld, Germany. I am one of the named inventors on European Patent Application 04 709 824.9, entitled "Diagnosis and treatment of multiple sulfatase deficiency and other using a formylglycine generating enzyme (FGE)".

2. I have been carrying out research in this field since 1995, when it first became known that a genetic defect of formylglycine generation led to multiple sulfatase deficiency, suggesting that FGE existed. My first publication in the field was in 1997 (Dierks, T., Schmidt, B. & von Figura, K. (1997) Conversion of cysteine to formylglycine: A protein modification in the endoplasmic reticulum. Proc. Natl. Acad. Sci. USA 94, 11963-11968), when we developed an assay for FGE activity, although we had not yet isolated the enzyme itself.

3. FGE was not isolated until 2003 (Dierks, T., Schmidt, B., Borissenko, L. V., Peng, J., Preusser, A., Mariappan, M. & von Figura, K. (2003) Multiple Sulfatase Deficiency is caused by mutations in the gene encoding the human Cα-formylglycine generating enzyme. Cell 113, 435-444), in research which led to the filing of the current patent application. It therefore took 6 years to go from an assay for FGE activity to isolating the enzyme itself.

4. During that time we developed the assay for FGE activity further, to arrive at a membrane-free assay. We also determined more precisely the peptide target sequence identified by the enzyme. The membrane-free assay is that described in Fey et al. (Characterization of posttranslational formylglycine formation by luminal components of

the endoplasmic reticulum, J. Biol. Chem. 276, 47021-47028; cited by the EPO examiner as D3), while the specific substrate target sequence is that described in Dierks, T., Lecca, M. R., Schlotterhose, P., Schmidt, B. & von Figura, K. (1999) Sequence determinants directing conversion of cysteine to formylglycine in eukaryotic sulfatases. EMBO J. 18, 2084-2091. The sequence given in Table 1 by Schirmer et al, cited by the EPO examiner as D2, reproduces the formylglycine modification sequence of arylsulfatase A as identified in Dierks et al. (1997) (see full reference in paragraph 2); this is correctly cited in D2. (So Schirmer was not the first to disclose this target sequence).

Isolation of the enzyme was not as simple as combining the assay of D3 with knowledge of the target sequence.

5. The source of material was one problem. We finally used bovine testis as starting material, from which we prepared by a laborious and newly developed protocol reticuloplasm, i.e. the soluble content of highly enriched microsomes. Although this contained relatively high levels of FGE activity, absolute FGE protein levels were low and highly diluted. It was necessary to purify FGE from ≥1 kg of testis tissue and to concentrate it some 20,000 times in order to obtain sufficient enzyme in a 50 μl volume suitable for analysis by gel electrophoresis, tryptic digestion and mass spectrometry.

6. A further problem was that the assay described in D3 was not practical to isolate the enzyme. The assay of D3 was relatively slow and laborious, taking around 5 days to process 10 samples. In order to analyze the numerous samples of the enzyme to be tested during each purification step, we had to modify the assay. In particular, we replaced the assay of D3 with a new assay based on a peptidic substrate and MALDI-TOF mass spectrometry. Without this new assay, we would not have been able to isolate the enzyme. The assay requires expert skills and till this day we are the only lab having successfully applied this assay to measure eukaryotic FGE activity.

7. Another major difficulty however was that when trying to isolate the enzyme using an affinity column, initially no FGE activity was isolated.

The affinity column utilized in our initial experiments made use of the substrate peptide ASA(65-80) (PVSLCTPSRAALLTGR), which is the sequence identified in Dierks et al. (1997) (see full reference in paragraph 2). We were not able to successfully isolate FGE activity with this column. Our initial hypothesis was that the enzyme was eluted from the column but was somehow inactivated. In fact, we later determined that the enzyme forms a covalent bond with the substrate cysteine, and does not elute from the column. To overcome this, it proved necessary to substitute this cysteine by serine, which proved to avoid the formation of covalent bonds, but still allowed the enzyme to recognise the target sequence. Neither D2 or D3 taught or suggested this substitution.

8. I would mention here that the substitution of cysteine by serine was not "to prevent the enzyme from releasing the substrate after the enzymatic reaction to increase the purification yield" (as suggested by the EPO examiner in his communication of 28 June 2006). Indeed, as was subsequently discovered, the problem was just the opposite, namely that the enzyme did not release the substrate; the two were covalently interconnected via a disulfide bridge, hence preventing enzyme elution and its detection through the activity assay. Had an attempt been made to prevent substrate release, this would have had no effect on the problem. The examiner also suggests in the same communication that substitution of cysteine by serine would be obvious as serine is "a known modification target of FGEs (see eg D2)". In fact, as D2 explains, there are no serine-specific FGEs in eukaryotes, only in prokaryotes. Further, D2 notes that replacement of the target cysteine in the human sulfatase enzyme ArsA with serine abolishes the target modification to FGly (D2 at page R181, paragraph bridging left and right columns), which actually had been shown, as correctly cited in D2, by Dierks et al. (1997) (see full reference in paragraph 2). The two forms of FGE (serine-specific and cysteine-specific) are completely different, and there would be no reason to expect that replacement of cysteine by serine in the substrate target sequence would be desirable for the affinity purification described in the present application. And yet, without that modification, isolation of FGE would not have been possible.

9. In the communication of 10 October 2007, the EPO examiner at item 4.2 states, "D2 clearly discloses the **human substrate peptide** which would be considered by the skilled person" (emphasis in the original). I disagree with this statement, because, as discussed in paragraph 8, use of the peptide given in D2 would not have led, and indeed did not lead, to the successful isolation of FGE.

10. The examiner goes on to further state:

> The only remaining and decisive question is whether the skilled person would construct a substrate affinity column using the human peptide sequence of D2, Table 1. If the skilled person would do so, he/she would isolate the FGE of the present application with a reasonable expectation of success, since no surprising technical effects associated with the FGE of the present application or any experimental difficulties can be derived from the application as filed. (Item 4.2)

I disagree with the examiner's conclusion. Without the serine for cysteine substitution described in paragraph 7, isolation of FGE would not have been possible.

11. In addition, I note that the examiner states at item 4.3 of the communication of 10 October 2007:

> Using the information at hand the skilled person knows that the enzyme will interact with the substrate. Thus, using the general background knowledge of a skilled person working in the field of enzymology the skilled person would of course immediately think about using enzyme-substrate affinity chromatography, a general technique well known since the late 1960's. The skilled person does not need a direct mentioning of this general technique in D2/D3 since the problem to be solved and the technical disclosure of D2 and D3 nearly inevitably lead to this technique because it is a well established straightforward strategy and alternatives can hardly be imagined using the information in the prior art.

The examiner underestimated the complexity of the procedure needed to isolate FGE. If a skilled person had used the substrate described in D2 to try to purify FGE, the person would not have succeeded in isolating the FGE. Further, as the examiner notes, based on the information in the prior art "alternatives can hardly be imagined." Clearly, the serine for

cysteine substitution needed to successfully isolate FGE was not taught or even suggested in the prior art. Yet as described in paragraph 7 and in the application (see, e.g., page 72, lines 28-29 of WO 2004/072275), this substitution was crucial for the elution of active FGE.

12. Further problems in the isolation of FGE remained even once FGE activity could be eluted from the column. We obtained many distinct protein species, but were unable to identify the enzyme, since no homologous sequences with an ascribed redox function were present in the databases at the time. We therefore developed three additional chromatographic isolation steps – ion exchange, concanavalin A, and negative selection using a mutagenized substrate peptide ligand – in order to eliminate other proteins from the eluate. In the final fraction, 5% of the starting FGE activity and 0.0006% of the starting protein were recovered, which corresponds to a 8,333-fold enrichment.

13. When we finally obtained a purified sample of the putative FGE, we identified two proteins, one of 39.5 kDa and one of 41.5 kDa. Mass spectrometry determined that these had largely overlapping sequences, but in the databases we could not identify any proteins of known function having that sequence. In particular, there was an absence of homology with the known prokaryotic enzymes having FGE function. Confirmation of FGE activity therefore required cloning of the gene (or corresponding cDNA) and expression in fibroblasts having multiple sulfatase deficiency to determine whether sulfatase function was restored.

14. Thus, to obtain the sequence of the isolated FGE took 6 years from initial development of an assay for FGE activity. The steps taken were not straightforward, and several problems had to be overcome in order to obtain the protein. In particular, the known assay for FGE was unsuitable for the task, such that a new assay had to be developed; and use of the known substrate target sequence in affinity chromatography was unable to isolate the enzyme. Further problems were a result of the absence of identifiable homologues to the enzyme in the databases, leading to difficulties in confirming that the isolated enzyme was indeed a novel eukaryotic FGE.

And I make this declaration believing that the facts in it are true.

Thomas Dierks

17-APR-2008

Date

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